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Sodium Fluoride (NaF) Releases the Two-Cell Block in Mouse Embryos

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ABSTRACT—This study was undertaken to elucidate the mechanism underlying the inability of mouse embryos to develop in culture past the 2-cell stage (2-cell block). In complete TLP-PVA medium, the rate of development of non-inbred (ICR) mouse 2-cell embryos to 3- and 4-cell stages (≧3 cells) was only 11%. However, sodium fluoride (NaF, an inhibitor of enolase in glycolysis) significantly improved the rate of further development of 2-cell embryos in a dose-fashion manner. NaF at 5 mM allowed 54% of 2-cell embryos to develop to ≧3 cells, although NaF at concentrations greater than 7.5 mM stunted their development. The removal of glucose and phosphate from the TLP-PVA medium improved the rate of development of 2-cell embryos to ≧3 cell (42%), but the rate of development of 2-cell embryos to ≧3 cells was enhanced (77%) when NaF at 2.5 mM was added to the medium without glucose and phosphate. The effect of NaF at 2.5 mM on $^{14}CO_2$ and [^{14}C]lactate production from [^{14}C]glucose was also examined. NaF at 2.5 mM significantly inhibited both $^{14}CO_2$ and [^{14}C]lactate production from [6-^{14}C]glucose, but not $^{14}CO_2$ production from [1-^{14}C]glucose, although it had no effect on the uptake of [^{3}H]deoxy-D-glucose (a non-metabolizable analogue of glucose) by the embryos. When the intracellular level of adenosine triphosphate (ATP) during early development of mouse embryos was examined, it was higher before rather than after cleavage. In addition, the ATP level was much higher before the 2nd cleavage 30 hr after insemination in unblocked embryos than that in blocked ones. These results suggest that the flow rate of glucose in glycolysis may regulate the development of 2-cell embryos to ≧3 cells, and that the intracellular level of ATP may be critical to early phase of mouse embryo development in vitro.

INTRODUCTION

Mouse embryos have been used for research on pre-implantation embryology. Successful *in vitro* culture of fertilized mouse eggs beyond the blastocyst stage would provide useful information on the early phases of development. However, with the exception of some inbred and F1 strains [12, 27], 2-cell embryos from non-inbred mice do not develop into 4-cells in a chemically defined medium (referred as "2-cell block"). This phenomenon is not restricted to the mouse. Cattle embryos cease development at the 8- to 16-cell stage [26], pigs at 4-cells [11] and hamsters at 2-cells and 4-cells [6]. However, this is not true of all mammalian species. The blocks do not occur in embryos of the rabbit [15], rhesus monkey [18], and humans [22]. The reasons for this difference still remains unclear. For development of 2-cell embryos into the blastocyst stage *in vitro*, various methods have been employed; determination of the optimal physicochemical culture environment including energy substrates [10], low oxygen concentrations [23], the removal of glucose and phosphate [24], and the addition of ethylenediamine-tetraacetic acid (EDTA) [1]. However, the cause(s) of the block still remains far from perfect understanding.

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Recently, we reported that approximately a half of the mouse 2-cell embryos developed to ≧3 cells *in vitro* by eliminating glucose and phosphate from the culture medium [3], although the mechanism for this is not clear yet. If considered that glucose is the substrate for glycolysis while phosphate stimulates three glycolytic enzymes hexokinase, phosphofructokinase, and glyceraldehyde-3-phosphate dehydrogenase, it is assumed that when glycolysis in mouse embryos is inhibited, the limitations imposed by the 2-cell block can be circumvented. This study was undertaken to examine whether the 2-cell block is cancelled when glycolysis is inhibited, using NaF which is reported to be an inhibitor of enolase [20]. Here we report that NaF released the 2-cell block in mouse embryos, and that the intra-cellular level of ATP may be critical in regulating the early phase of embryo development.

MATERIALS AND METHODS

Collection and culture of embryos

In all experiments, random-bred ICR male and female mice (10–12 weeks old) were used. ICR mice were super-ovulated with intra-perioneal injections of 5 i.u. PMSG (Teikoku Zoki) at intervals 48 hr apart. At 16–17 hr post-hCG, the females were killed by cervical dislocation, the oviducts dissected out and the cummulus masses released into a TLP-PVA medium which contained 0.35 mM phosphate, 2.8 mM glucose and 4 mg/ml bovine serum albumin (BSA) [5]. For fertilization in vitro, male mice were killed 14 hr after the females were injected with hCG, the vasa deferentia and epididymides dissected out and one of each placed in a 0.4 ml drop of pre-gassed TLP-PVA medium with 4 mg BSA/ml. The sperm were squeezed out gently and allowed to capacitate for 2 hr at 37°C. At 18–19 hr post-hCG, 10 μl of the sperm suspension was added to each 0.4 ml drop of the TLP-PVA medium in a Falcon 35-mm dish (Falcon) to give a final sperm concentration of 150 sperm/μl. Eggs and sperm were incubated together for 5 hr at 37°C under liquid paraffin oil (Squibb). To minimize damage to embryos prior to culture, oviduct dissection and collection and placement of embryos into culture were carried out as soon as possible. After 5 hr incubation, the eggs were washed twice with a fresh medium pre-gassed with 5% CO_2 in air at 37°C, transferred into 0.4 ml of the conditioned medium covered with liquid paraffin oil, and incubated at 37°C under 5% CO_2 in air for certain periods of time.

Experimental studies

Experiment 1: Concentrations (0, 0.5, 1.0, 2.5, 5, 7.5 and 10 mM) of NaF were tested for their effects on development of 2-cell embryos to ≧3 cells in vitro.

Experiment 2: Lacking 2.8 mM glucose and 0.35 mM phosphate, but with 2.5 mM NaF, in the TLP-PVA medium, EDTA (100 μM), which is known to cancel the 2-cell block [14], was tested to see its effect on the rate of 2-cell embryo development beyond the 2-cell stage.

Experiment 3: In conjunction with the NaF effect on the development of 2-cell embryos beyond the 2-cell stage, production of $^{14}CO_2$ from [1-^{14}C]-glucose (0.29 GBq/mmol; Amersham) and [6-^{14}C]glucose (0.26 GBq; Amersham) by 2-cell embryos was determined in the presence or absence of 2.5 mM NaF in a medium without glucose and phosphate. For determination of $^{14}CO_2$ production, fifty 2-cell embryos/tube were collected 24 hr after insemination and incubated with [^{14}C]glucose (0.6 nmole/tube) for 3 hr at 37°C in 0.5 ml of the TLP-PVA medium in a vessel capped tightly with a rubber stopper. Incubation was terminated by injection of 0.5 ml of 50% citric acid (w/v) through the rubber stopper [21]. The $^{14}CO_2$ liberated from [^{14}C]glucose was trapped in KOH and its radioactivity was determined by a liquid scintillation spectrometer (Aloka, model ISC-903) [21].

Experiment 4: [^{14}C]Lactate production from [^{14}C]glucose by 2-cell embryos was determined using the method of Kusaka and Ui [17]. Fifty of 2-cell embryos/tube were collected 24 hr after insemination, and incubated with [6-^{14}C]glucose (0.6 nmole/tube) for 3 hr at 37°C in 0.5 ml of the medium. Incubation was stopped by the addition of 0.5 ml of 60% perchloric acid to the medium. Two tenths of a ml of each sample was taken and 1.9 ml of water was added before centrifugation.

The supernatant was added with 10 mg of semicarbazide (Wako) and 20 mg of charcoal (Wako), and kept for 15 min at 0°C with occasional shaking before centrifugation. One and a half ml of the resultant supernatant was introduced into 0.5 ml of 0.5 M glycine solution (pH 9.5) containing 0.28 M semicarbazide, 3 mg EDTA (Sigma), and 2 mg NAD (Boehringer Mannheim). The reaction was started by the addition of 18 units of lactic dehydrogenase (Boehringer Mannheim), then allowed to proceed for 90 min at 25°C before being terminated by lowering the pH below 3 with cold HCl. Seventy mg of charcoal was then added to the solution and the sample was kept at room temperature for 30 min with occasional shaking. Charcoal, separated by centrifugation, was washed twice with water and eluted with a mixture of 40% methanol/20% ethanol/0.8% NH_4OH. After 30 min, 4 ml of the effluent was evaporated to dryness. The resulting residue which was then dissolved in a small amount of water was applied to a thin layer plate of cellulose (Funakoshi), and developed with the solvent system of isopropanol/formic acid/water (6:1:1) [17]. The radioactivity of the spot of pyruvic semicarbazone detected under a UV lamp was counted in a liquid scintillation counter (Aloka, model ISC-903) [21].

Experiment 5: The uptake of [^{3}H]deoxy-D-glucose by 2-cell embryos was determined. Fifty 2-cell embryos/tube were collected 24 hr after insemination, and incubated with 1 nmole/tube of 2-[2,6-^{3}H]deoxy-D-glucose (1.63 TBq/mmol; Amersham) for 3 hr at 37°C in 0.5 ml of the TLP-PVA medium with or without NaF at 2.5 mM, and without glucose and phosphate. After incubation, the embryos were washed 5 times with fresh medium and their radioactivity was determined [21].

Experiment 6: Determination of intra-cellular levels of ATP was carried out. After 150–200 2-cell embryos/point were incubated at 37°C for certain periods of time in the TLP-PVA medium, (with or without 100 μM EDTA), 40 μl of 9% perchloric acid were added. The sample was kept at 0°C for 30 min, and then 16 μl of 2 M K_2CO_3 solution was added. The ATP level in the embryos was determined using the method of Singer et al. [25] using a LKB luminometer (Pharmacia, model 1250).

Evaluation of embryos and statistical analysis

Embryos were evaluated according to stage of development by counting the number of 1-, 2-, 3- and 4-cell embryos after certain periods of time of culture under a stereomicroscope (Nikon, model SMZ-1). Paired comparisons were made to determine significance.

RESULTS

Effects of NaF, glucose and phosphate on development of 2-cell embryos into 3- and 4-cell ones

NaF was added to the culture medium and its effect on the development of 2-cell embryos to ≧3 cells was examined. As shown in Fig. 1, NaF significantly cancelled the 2-cell block in a dose-fashion manner. The rate of development of 2-cell embryos beyond the 2 cell stage reached a maximum at 5 mM NaF. At 2.5 mM and 5 mM NaF, 47% (194/411) and 54% (86/160) of 2-cell embryos developed to ≧3 cells, respectively, while

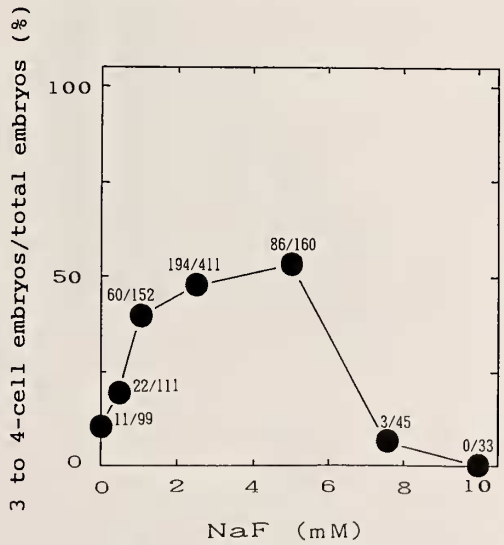

Fig. 1. The effect of NaF on the development of 2-cell embryos beyond the 2-cell stage.
Five hr after insemination, embryos were transferred to the pre-gassed fresh medium containing various concentrations of NaF and incubated at 37°C for another 43 hr. The embryo stages were then evaluated under stereomicroscopy.

without NaF only 11% (11/99) of 2-cell embryos did. However, NaF at concentrations greater than 7.5 mM stunted their development. We thus used 2.5 mM NaF for further studies. When glucose and phosphate were omitted from the culture medium, 42% (44/106) of 2-cell embryos went through a 2nd cleavage. Development of 2-cell embryos to ≧3-cells in the medium containing 2.5 mM NaF, 2.8 mM glucose and 0.35 mM phosphate (56%) was significantly higher ($P<0.05$) than in the medium without glucose and phosphate (42%) (Fig. 2). When 2.5 mM NaF was added to the medium without glucose and phosphate, 77% (142/184) of 2-cell embryos underwent a 2nd cleavage. This rate was quite similar to that (80%) of the development of 2-cell embryos treated with 100 μM EDTA (Fig. 2).

Production of $^{14}CO_2$ and [^{14}C]lactate from [^{14}C]-glucose by 2-cell embryos

$^{14}CO_2$ and [^{14}C]lactate production from [^{14}C]glucose was determined to see whether glycolysis in mouse embryos was inhibited by NaF. Two-cell embryos were collected 24 hr after insemination and incubated for 3 hr at 37°C in the presence or absence of 2.5 mM NaF in the medium without glucose and phosphate. As shown in Fig. 3, there was no difference ($P>0.5$) in $^{14}CO_2$ production from [1-^{14}C]glucose between mediums with or without NaF, but there was a significant difference ($P<0.01$) in $^{14}CO_2$ production from [6-^{14}C]glucose. In addition, NaF inhibited [^{14}C]-lactate production from [6-^{14}C]glucose by approximately 50% ($P<0.01$) when compared to the control embryos (Fig. 4).

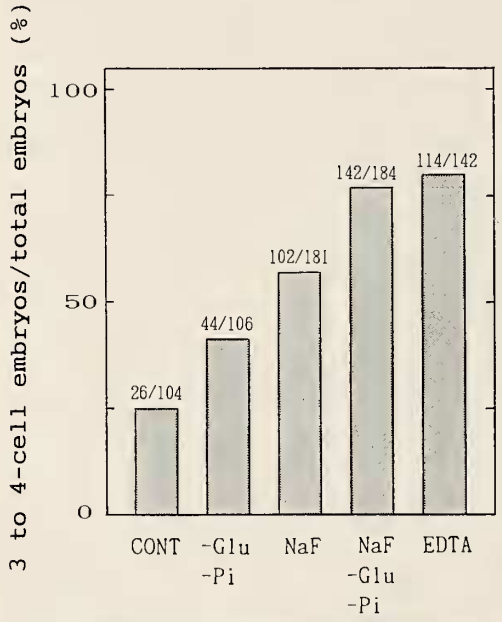

FIG. 2. Effects of NaF, glucose and phosphate on the release of 2-cell block.
Five hr after insemination, embryos were transferred to the fresh TLP-PVA medium with or without 2.8 mM glucose, 0.35 mM phosphate, 2.5 mM NaF and 100 μM EDTA. Embryos were then incubated for another 43 hr at 37°C and the ratio of 3- and 4-cells/total cells (%) was obtained by counting 3- and 4-cell embryos under stereomicroscopy.

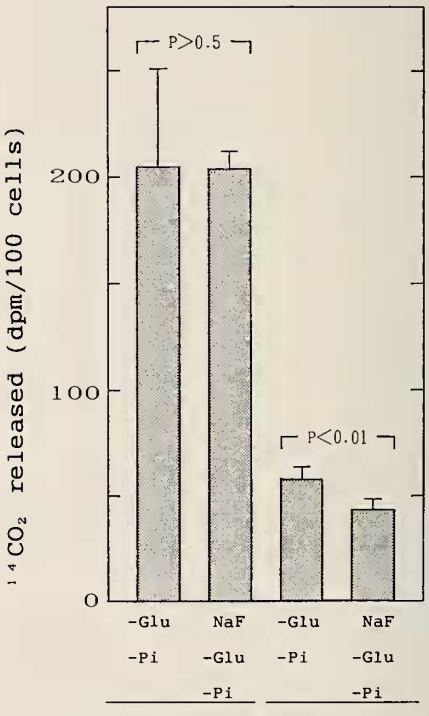

FIG. 3. $^{14}CO_2$ productiuon from [1-^{14}C]- and [6-^{14}C]-glucose.
$^{14}CO_2$ production from [1-^{14}C]- and [6-^{14}C]glucose was determined using the method as described in "Materials and Methods". $P>0.5$ and $P<0.01$; compaired with 2.5 mM NaF.

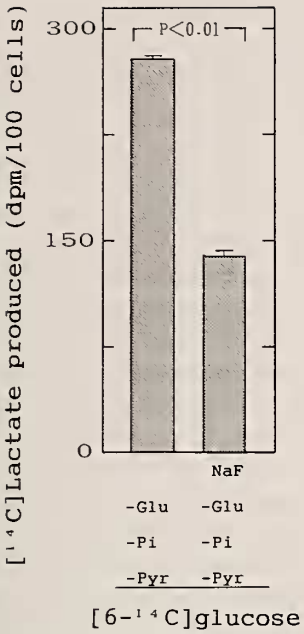

FIG. 4. [^{14}C]-Labeled lactate production from [6-^{14}C]glucose by 2-cell embryos.
Production of [^{14}C]lactate from [6-^{14}C]glucose by 2-cell embryos was determined as described in "Materials and Methods". $P<0.01$; compaired with the addition of NaF.

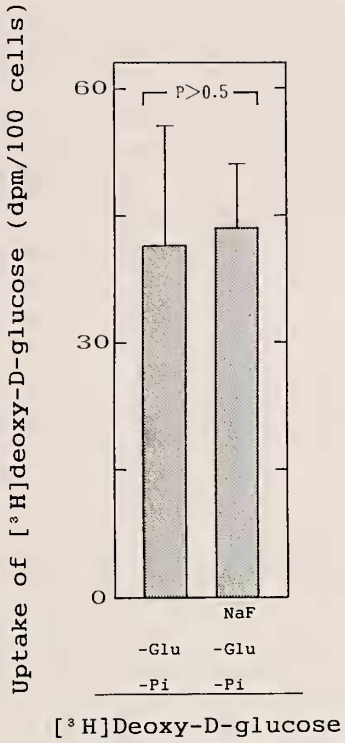

FIG. 5. Uptake of [^{3}H]deoxy-D-glucose by 2-cell embryos.
The [^{3}H]deoxy-D-glucose uptake by 2-cell embryos was determined as described in "Materials and Methods". $P>0.5$; compaired with NaF.

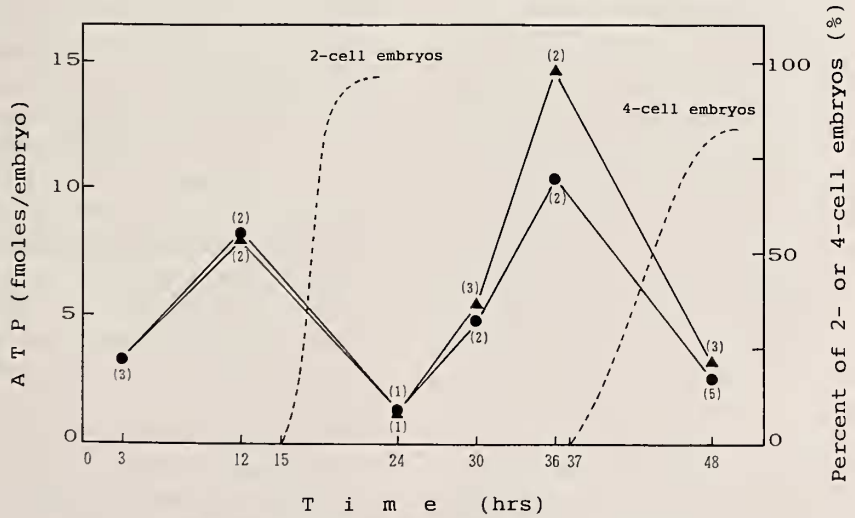

FIG. 6. Intra-cellular level of ATP during the developmental stages of mouse embryos.
Embryos were incubated with or without 100 μM EDTA for certain periods of time at 37°C and the ATP levels in the embryos were determined as described in "Materials and Methods". Dotted lines indicate the rate of embryos undergoing first or second cleavage per total embryos. To obtain these ratios, at least 50 embryos were counted under stereomicroscopy every 1 hr during the development of embryos in vitro. Time 0 means insemination time. The number of ATP determinations is indicated in parenthesis.

[^{3}H]Deoxy-D-glucose uptake by 2-cell embryos

Since NaF inhibited the glycolytic system of mouse 2-cell embryos, we examined whether the reduced production of $^{14}CO_2$ and [^{14}C]lactate from [6-^{14}C]glucose was due to inhibition of glucose uptake by 2-cell embryos by NaF. However, NaF at 2.5 mM had no effect on the uptake of [^{3}H]deoxy-D-glucose by 2-cell embryos ($P>0.5$) (Fig. 5).

ATP determination

The ATP level in embryos was determined when they were treated with or without 100 μM EDTA. As shown in Fig. 6, the first and 2nd cleavages occurred at 15–22 hr and 37–48 hr after insemination, respectively. The ATP level in embryos was found to be higher prior to the cleavage. When embryos were treated with or without 100 μM EDTA, there was no difference in the ATP level 12 hr before the first cleavage between two groups, but there was a difference in the ATP level 30 hr before the 2nd cleavage. The ATP level in embryos treated with EDTA was much higher than that in untreated ones (14.6 ± 0.6 vs 10.4 ± 2.2 fmoles/cell; $n=2$).

DISCUSSION

This study clearly showed that NaF enhanced the development of mouse 2-cell embryos into the 3- and 4-cell ones. To the best of our knowledge, this is the first report indicating that treatment of mouse embryos with NaF significantly releases the 2-cell block. We have previously reported that the removal of glucose and phosphate from the culture medium improved the rate of development of mouse 2-cell embryos to $\geqq$3 cells (42%) [3], which was consistent with the results obtained in this study. However, development of 2-cell embryos to $\geqq$3 cells in the medium containing NaF, glucose and phosphate was significantly more enhanced than in a medium free of glucose and phosphate (see Fig. 2), whereas only 11% of 2-cell embryos developed to $\geqq$3 cells in the complete culture medium without NaF. It is of greatly interest to note that when cultured in a medium without glucose and phosphate, but with NaF, approximately 80% of 2-cell embryos underwent a 2nd cleavage. This rate was quite similar to that in the development of 2-cell embryos cultured in the presence of EDTA.

Inhibition of the development of mouse embryos by glucose may be attributable to the Crabtree effect; (a) inhibition of respiration and oxidative phosphorylation by glucose [9], and (b) the fact that phosphate becomes rate-limiting for oxidative phosphorylation and respiration in the presence of glucose [16]. Previous studies by Aoki *et al.* [3] showed that inhibition of embryo development by glucose occurred both in the presence and absence of phosphate, and that the reverse was also true; embryo development was inhibited both in the presence and absence of phosphate when glucose was present. By this, an inadequate ATP production would occur. Glucose is a substrate for glycolysis, whereas, as mentioned before, phosphate stimulates glycolytic enzymes. Intra-cellular phosphate may thus be utilized mainly for glycolysis, with consequent disruption of phosphorylation reactions in mitochondria (low production of ATP). As a result, poor or blocked embryo development would occur. This is supported by the fact that the ATP level in blocked embryos was lower than that in normally developed ones. When glucose is removed from the culture medium, phosphate could enhance the breakdown of stored glycogen to form glucose-6-phosphate. When concentrations of glucose-6-phosphate are high, glycolysis would be stimulated, resulting in insufficient ATP production and inhibition of embryo development. However, when phosphate and glucose concentrations are lowered, the glycolytic system would be suppressed, resulting in high ATP production in mitochondria. These explanations for the observed inhibitory effect of glucose and phosphate on embryo development are consistent with the fact that NaF, an inhibitor of the glycolytic enzyme, improved the rate of development of mouse 2-cell embryos to $\geqq$3 cells. The suppression of glycolysis by NaF is not due to a reduced uptake of glucose, since this drug had no effect on the uptake of [^{3}H]deoxy-D-glucose.

From studies of pre-implantation mouse embryos [8], glucose is known to be an unfavorable energy source for development until the 8-cell

stage. The reason for this failure of early-stage mouse embryos to develop on glucose alone is found in the block at the phospho-fructokinase step in glycolysis [4]. This conclusion is based on the finding that maximum glucose-6-phosphate and fructose-6-phosphate levels are much lower during the early stages (2- and 8-cell stages) than at later stages (morula and blastocyst stages), suggesting that glycolysis activity in mouse embryos is low. However, embryos used for such studies [4] were developed *in vivo* in the reproductive tract where the 2-cell block did not take place, and so were obtained by being flushed from the tract. If the glycolysis of embryos in the reproductive tract is inhibited by a factor(s), which is unknown at the present time, or is suppressed because of, for example, low concentrations of glucose, the levels of glucose-6-phosphate and fructose-6-phosphates would be low. When, in fact, free sugars in rabbit oviduct were tested for glucose, fucose, xylose, sorbose, galactose, fructose and mannose, glucose, in trace amounts, was the only free sugar found [7, 13]. This might be one of reasons why the 2-cell block does not occur *in vivo*. In culturing mouse 2-cell embryos *in vitro*, the glycolytic system is probably not inhibited, presently because of the differing environments. Consequently, they cannot develop to $\geqq 3$ cells, but can do so when the glycolysis in 2-cell embryos is inhibited by NaF. However, this is too speculative. To prove this hypothesis, the level of glycolytic metabolites in embryos must be determined when cultured in the medium with or without NaF.

We cannot exclude the possibility that NaF, which is also known as an inhibitor of protein phosphatase [19], could release the 2-cell block by the mechanism other than inhibiting the glycolysis in mouse embryos. Recently, Aoki *et al.* [2] reported that the phosphorylation followed by dephosphorylation of ${}_{p}34^{cdc2}$ is required for mouse 2-cell embryos to develop to $\geqq 3$ cells. In their studies [2], an inhibitor of protein phosphatase I and 2A, ocadaic acid, dephosphorylated ${}_{p}34^{cdc2}$ in the mouse embryos arrested at the 2-cell stage. It is, therefore, conceivable that NaF could also dephosphorylate ${}_{p}34^{cdc2}$, resulting in the release of the 2-cell block. The low level of intra-cellular ATP in the blocked embryos may fail in the phosphorylation of ${}_{p}34^{cdc2}$. Further studies must be carried out to answer these questions. Nevertheless, it is of great interest to note that the development of mouse 2-cell embryos to $\geqq 3$ cells was significantly enhanced when glycolysis is inhibited by NaF. The intra-cellular level of ATP may be critical in regulating the early phase of embryo development in mice.

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